EXHIBIT II

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Cloning of a human tRNA isopentenyl transferase

Anna Golovko, Göran Hjälm[1], Folke Sitbon, Björn Nicander *

Department of Plant Biology, SLU, PO Box 7080, SE-75007 Uppsala, Sweden

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Abstract

A cDNA of human origin is shown to encode a tRNA isopentenyl transferase (E.C. 2.5.1.8). Expression of the gene in a *Saccharomyces cerevisiae* mutant lacking the endogenous tRNA isopentenyl transferase MOD5 resulted in functional complementation and reintroduction of isopentenyladenosine into tRNA. The deduced amino acid sequence contains a number of regions conserved in known tRNA isopentenyl transferases. The similarity to the *S. cerevisiae* MOD5 protein is 53%, and to the *Escherichia coli* MiaA protein 47%. The human sequence was found to contain a single C2H2 Zn-finger-like motif, which was detected also in the MOD5 protein, and several putative tRNA transferases located by BLAST searches, but not in prokaryotic homologues.

Keywords: Complementation; Cytokinin; Isopentenyladenosine; Suppressor tRNA; tRNA modification

1. Introduction

The cytokinins constitute a group of modified adenines with two important and apparently unrelated functions in nature. They are found as modified nucleosides in the tRNA of plants, animals and eubacteria but not in Archaea (Persson et al., 1994). In plants they also act in tRNA-free form as hormones to regulate cell division, shoot morphogenesis, chloroplast maturation and many other developmental processes (Mok, 1994). Even though isopentenyladenosine (i^6A), the only cytokinin of animal tRNA, has been found in tRNA-free form in cell cultures (Adair and Brennan, 1986), no physiological role in animals has been demonstrated. However, the addition of cytokinins was shown to affect cell culture growth rate (Gallo et al., 1969) and DNA synthesis (Quesney-Huneeus et al., 1980) in animals.

Cytokinins in tRNA are situated at position 37, next to the anticodon, of certain tRNAs that bind to codons starting with a U. The major form in *Escherichia coli*, 2-methylthiolated i^6A, plays an important role in translational efficiency and fidelity (Persson et al., 1994).

The first step in the biosynthesis of tRNA cytokinins is the transfer of an isopentenyl group from dimethylallyl pyrophosphate to A37 of the preformed tRNA. The reaction is catalyzed by tRNA isopentenyl transferase (E.C. 2.5.1.8), termed IPT in this study. This enzyme has been purified from *E. coli* (Rosenbaum and Gefter, 1972; Leung et al., 1997; Moore and Poulter, 1997), *Zea mays* (Holtz and Klämbt, 1978) and *Saccharomyces cerevisiae* (Kline et al., 1969). IPT genes have been cloned from several microorganisms, including *miaA* from *E. coli* (Caillet and Droogmans, 1989; Connolly and Winkler, 1989) and *Agrobacterium tumefaciens* (Gray et al., 1992), and *MOD5* from *S. cerevisiae* (Dihanich et al., 1987). An additional number of putative IPT genes from various organisms can be found by BLAST searches on various gene databases. A comparison of the sequences of identified and putative tRNA isopentenyl transferase genes suggests that they constitute a family of genes highly conserved in evolution (Tolerico et al., 1999). Several sequences homologous to IPT were found by BLAST searches in the human dbEST database. We report here the identification of one of these as coding for an enzyme with IPT activity, the first member of this gene family identified from a multicellular organism.

Abbreviations: hMOD5, *Homo sapiens* IPT; IPT, tRNA isopentenyl transferase; MOD5, *Saccharomyces cerevisiae* IPT.

* Corresponding author. Tel.: +46-18673324; fax: +46-18673279.
E-mail address: bjorn.nicander@vbiol.slu.se (B. Nicander)
[1] Present address: Uppsala University, Department of Medical Biochemistry and Microbiology, Box 582, SE-751 23 Uppsala, Sweden.

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2. Materials and methods

2.1. Strains and transformation

The *S. cerevisiae* strains used were MT-8 (*MATα SUP7 ura3-1 his5-2 leu2-3,112 ade2-1 trp1 lys1-1 lys2-1 can1-100 mod5::TRP1*) (Gillman et al., 1991) and H57 (*MATα MOD5 SUP7 can1-100 ade2-1 his5-2 lys1-1 ura3-1*) (gift from A. Hopper). Yeast strains were maintained on YPD medium (Sherman, 1991). The cells harboring the pFL61 plasmids were grown in synthetic complete medium (SC) (Sherman, 1991) lacking uracil (SC − ura). For marker selection, the media used was SC lacking the appropriate nutrient, or supplemented with 60 μg/ml canavanine (Sigma). Yeast cells were transformed using the LiOAC method as described (Gietz and Schiestl, 1997). Recombinant DNA was propagated in the *E. coli* strain DH5α using standard techniques (Sambrook et al., 1989).

2.2. Plasmid constructions

EST clones investigated for homology to yeast IPT had accession numbers AA356092, F07677, AA322152, AA309660, AA204763 and HE8AZ89. The last clone was obtained from the TIGR/ATCC special collection of human cDNA clones (Adams et al., 1995), and used in the following. An unspliced intron in this cDNA was removed as follows. Total RNA was extracted from human kidney using the TRIzol reagent (Life Technologies Inc.), following the manufacturer's instructions. Oligo-d(T)-primed first-strand cDNA synthesis was performed on 3 μg total kidney RNA in a 20 μl reaction with 200 U of SuperScript II RT (Life Technologies Inc.) as described by the manufacturer. PCR was performed on aliquots of this reaction with the sense primer (5'-CAACTGCTCTGATTG-AAGAT-3', position 329–349 of the cDNA), and the antisense primer (5'-TCCGCATAGCACTCCTTTG-3', position 1613–1595) in 40 μl reactions containing 0.5 μM of each primer, 50 μM dNTP, 1.3 mM $MgCl_2$ 1 × II Ampli*Taq* Gold buffer, and 0.15 U Ampli*Taq* Gold (PE Biosystems, Foster City, CA, USA). PCR cycling parameters were: heat-activation of the enzyme at 95°C for 12 min; 42 cycles of 94°C for 15 s, 52°C for 10 s, and 72°C for 90 s. This protocol yielded a single 1.2 kb PCR product, which was cloned and sequenced.

Clone HE8AZ89 was digested with *Eco*130I and *Mun*I (MBI Fermentas, Vilnius, Lithuania), resulting in cleavage at positions 652 and 942 respectively. The excised fragment was replaced by a fragment generated in a corresponding manner from the 1.2 kb PCR product. The integrity of the reconstructed cDNA sequence in HE8AZ89 was confirmed by DNA sequencing.

This sequence of the human tRNA isopentenyl transferase has GenBank accession number AF074918.

The entire predicted open reading frame (ORF) of the cDNA from the reconstructed HE8AZ89 was PCR-amplified using primers hMOD5.s (5'-TAGAAGCGGCCGCGAATTCGGCACGAGCTGCCATAA-3') and hMOD5.as (5'-GATAAAGCGGCCGCGAATTCCG-AGAACTAGTTTGGTTCA-3'; *Not*I sites underlined). The resulting PCR product was digested with *Not*I and ligated into the *Not*I site of pFL61 (Minet et al., 1992), creating plasmid pFL61–hMOD5 such that expression of the ORF was under the control of the *S. cerevisiae PGK* promoter.

DNA sequencing was performed using the dRhodamine terminator chemistry kit (PE Biosystems). Sequence analysis was performed using the MacVector (Oxford Molecular, Oxford, UK), Factura, Auto-Assembler, and EditView (Perkin Elmer) software packages. The multiple sequence alignment was established with the ClustalW algorithm (Thompson et al., 1994) using MacVector. EST clones investigated for homology to yeast MOD5 had accession numbers AA356092, F07677, AA332152, AA309660, and AA204763.

2.3. 5' rapid amplification of cDNA ends (5' RACE)

RACE was conducted using a 5' RACE kit (Life Technologies Inc.) as described in the supplier's protocol. Briefly, total RNA was extracted from the human monoblast cell line U97 and first-strand cDNA synthesis was performed on the poly(A)+ fraction using the human IPT cDNA antisense primer RACE1 5'-TTTCTGGGTCCACCTGGCTTAG-3' (position 527–506, Fig. 1) and 200 U Superscript II reverse transcriptase. A mixture of RNase H and RNase T1 was added to remove the mRNA strand. The 3'-end of the cDNA was tailed with dCTP using terminal deoxynucleotidyl transferase. A first round of PCR was performed on the tailed cDNA template using the anchor primer provided by the kit and the antisense primer RACE2 5'-TCCAGAGCAGAGATTCAATG-3' (position 404–385, Fig. 1). A second round of PCR was performed on a 1:100 dilution of the amplification products using a second anchor primer from the kit (overlapping the first one: 5'-GGCCACGCGTCG-ACTAGTAC-3') and antisense primer RACE2. The resulting 439 bp PCR product was cloned and sequenced.

2.4. tRNA isolation

Yeast cells were grown at 30°C to mid-log phase in liquid SC medium or SC − ura medium for plasmid maintenance. Cells were collected by centrifugation, frozen in liquid nitrogen, and stored at −80°C until extraction. The cell pellets (1.5 to 2 g) were suspended in 30 ml ice-cold buffer containing 0.15 M NaCl, 50 mM NaAc, 10 mM MgAc and 6% SDS, pH 4.5, and soni-

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cated in the presence of 1 g of 0.2 mm glass beads. The extracts were shaken with an equal volume of phenol, and tRNA in the aqueous phases was purified from by LiCl precipitation and DEAE-cellulose chromatography (Buck et al., 1983).

2.5. Analysis of isopentenyladenosine

tRNA was digested with nuclease P1 (Roche Molecular Biochemicals) followed by bacterial alkaline phosphatase (Sigma) as described (Gehrke et al., 1982), except that the nuclease P1 incubation time was extended to 14 h.

High performance liquid chromatography (HPLC) separations were performed using a system from Gilson (Middleton, WI, USA) on a 250 × 4 mm C8 RP-select B column (Merck, Darmstadt, Germany). A gradient from 0.25 M ammonium acetate (pH 6.0) to a mixture of 40% acetonitrile (Rathburn, Walkerburn, Scotland, UK) in water was used (Buck et al., 1983). The flow rate was 1.5 ml/min. The effluent was monitored by scanning between 250 and 300 nm using a UV 3000 detector (TSP, Riviera Beach, FL, USA) and λ_{max} was determined by use of the PC1000 software from TSP.

To remove salts, peak material was evaporated and injected into a 125 mm column of the same type as above, and eluted with a 12 min linear gradient from water to 40% acetonitrile. Material from the HPLC peak was evaporated, an aliquot removed, mixed with 10 pmol of $^{2}_{5}$H-i^{6}A and analyzed in a Q-TOF tandem mass spectrometer (Micromass, Manchester, UK). In scanning mode, the amount of i^{6}A was estimated from the ratio between the molecular masses (336.2 and 341.2). The first analyzer was then locked on the MW (±2 Da), and in turn these molecules were fragmented in a collision chamber and the ions subsequently analyzed in the second mass analyzer.

3. Results and discussion

3.1. Cloning and sequencing of the human MOD5-like cDNA

The amino acid sequence of the tRNA isopentenyl transferase (IPT) MOD5 of *S. cerevisiae* was used to search the dbEST division in GenBank. Five ESTs of human origin with high similarity scores were found, originating from Jurkat T-cells, brain, embryo, Jurkat T-cells, and hNT neurons. Their nucleotide sequences strongly suggested that they were derived from the same gene, and the longest cDNA was chosen for further analysis. This cDNA clone, HE8AZ89, was obtained from the TIGR/ATCC special collection of human cDNA clones (Adams et al., 1995).

DNA sequencing showed the cDNA to be 2212 base pairs in length, containing two long ORFs separated by a short stretch with three in-frame stop codons. To examine if this stretch corresp nded to an unspliced intron, PCR was perf rmed using oligonucleotides positioned upstream and downstream of this area n human kidney cDNA. DNA sequencing of the product yielded a single continuous ORF, lacking 87 bp at the position of the putative intron, confirming the presence of an unspliced intron in clone HE8AZ89. The clone was then reconstructed using appropriate restriction enzymes to excise a 290 bp stretch with the entire intron, and replacing this with a fragment excised in a corresponding way from the human kidney PCR product (see Section 2 and Fig. 1).

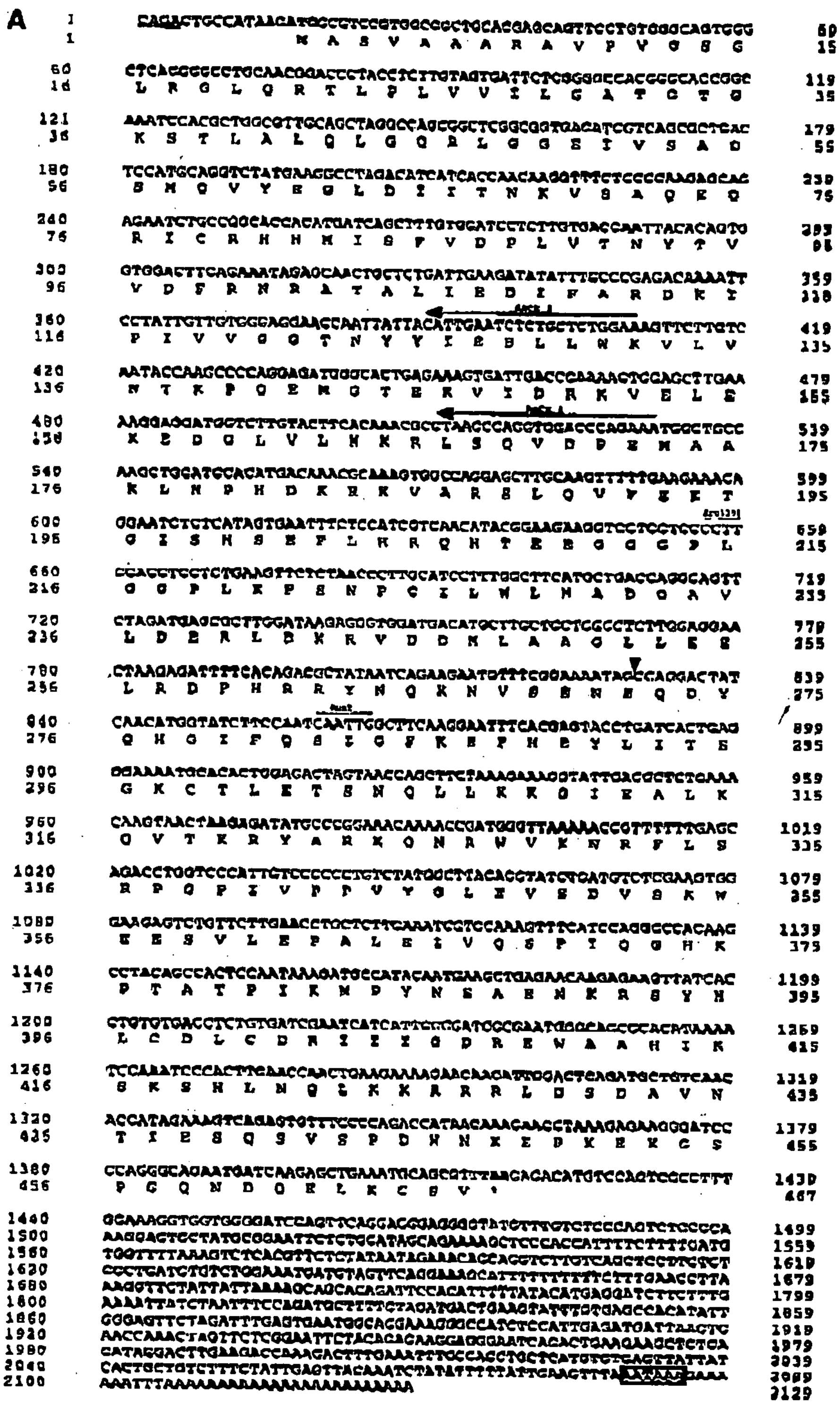
The cDNA was used to probe human monoblast cell mRNA, showing the presence of a single transcript approximately 2.2 kb in size (not shown). The 5′-end of the mRNA was mapped using 5′-RACE. This analysis indicated that the authentic transcript was four bases longer than the cDNA. Fig. 1 shows the 2129 nt long corrected cDNA. The predicted ORF consists of 1404 bp and encodes a protein of 467 amino acids with a calculated molecular weight of 52 721 Da.

3.2. The human MOD5-like cDNA complements a yeast mutant that lacks MOD5

In the yeast strain MT-8 the *MOD5* gene is completely inactivated by a *TRP1* insertion (Gillman et al., 1991). This prevents the i^{6}A modification of cytoplasmic and mitochondrial tRNAs, including the nuclear-encoded suppressor tRNA *SUP7* (Gillman et al., 1991). The lack of the i^{6}A modification renders *SUP7* unable to suppress certain nonsense mutations, such as those in the *ade2-1*, *can1-100* and *lys2-1* alleles (Zoladek et al., 1995). Cells failing to suppress the *ade2-1* mutation cannot grow on media lacking adenine and accumulate a red pigment when grown on rich media. The cells unable to suppress the *can1-100* mutation lack arginine permease and are able to grow in the presence of canavanine, a toxic analog of arginine.

To determine whether the human cDNA could complement the loss-of-suppression phenotype of MT-8, the cDNA-bearing construct pFL-hMOD5 was introduced. Table 1 shows the growth patterns on selective media. Adenine and lysine independence were restored, but only a slight growth inhibition on canavanine was seen. Cells transformed with the plasmid without insert were indistinguishable from untransformed cells with respect to *ade2-1*, *can1-100*, and *lys1-1/lys2-1* suppression (Table 1).

To determine if these growth patterns correlated with the synthesis of i^{6}A, total tRNA was extracted and enzymatically degraded. Fig. 2 shows HPLC separations of the resulting nucleosides. MT-8, lacking a functional *MOD5* gene, gave no peak eluting at the position of



B 826 ACATGAAATGGCTCAACTAGGTCCATCTCCAAAGCCCCTGCTAGCTTTTAGGAGCAATGGCTT 890
R * N G S T R G I S K A P A S F * E Q W L

891 ATCTGCTGATACCCTCCTGTTCCCAG 916
I C * Y P P V P

Fig. 1. Sequence of the human IPT cDNA. (A) Nucleotide and predicted amino acid sequences of human IPT. Amino acids positions were numbered from the putative translation initiation codon at nucleotide position 15. The vertical arrowhead denotes the position of an unspliced intron in the original EST clone HE8AZ89. Primers used in 5′ RACE PCR are indicated by arrows. The 5′-proximal bases determined by 5′-RACE PCR are underlined. The putative polyadenylation signal is boxed. Two restriction sites for the enzymes used to reconstruct HE8AZ89 are indicated. (B) Sequence of the unspliced intron in HE8AZ89. The splice sites are shown in bold. The asterisks denote the three in-frame stop codons of the encoded amino acid sequence. Italics denote the 3′ border of the upstream exon.

Table 1
Suppressor efficiency of *SUP7* in H57 and MT-8 strains

Strain	Suppression of *ade2-1*		Suppression of *lys1-1*, *lys2-1*	Suppression of *can1-100*
	Growth on −ade	Color on YPD	Growth on −lys	Growth on +can −arg
H57 (MOD5)	+	white	+	−
MT-8 + pFL61	−	red	−	+
MT-8 + pFL61–hMOD5	+	white	+	+/−[a]

[a] Poor growth compared with MT-8 + pFL61.

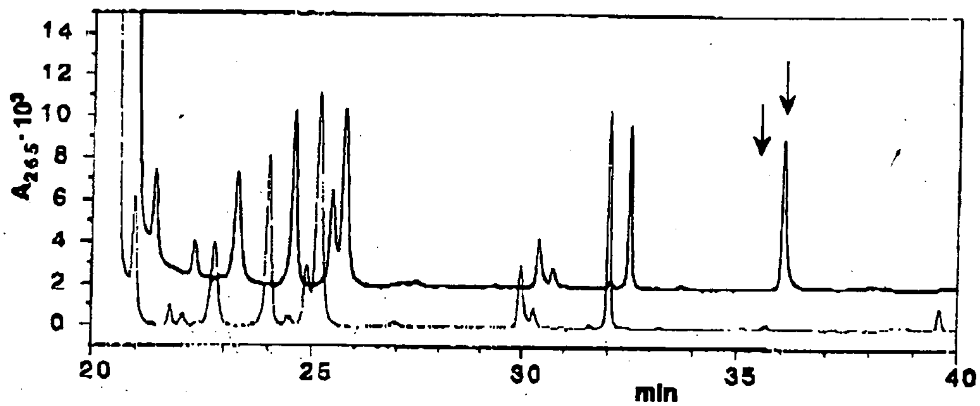


Fig. 2. Reversed-phase HPLC chromatograms of yeast tRNA modified nucleosides. Ca 4 OD_{260} units of tRNA were enzymatically digested to nucleosides and injected. The top trace is shown with a 1 min delay for clarity. Only the parts of the chromatograms containing the more hydrophobic nucleosides are shown. The off-scale peak at 20 min is adenosine, present at levels far exceeding those of the modified nucleosides. Bottom trace: tRNA nucleosides from MT-8 transformed with plasmid pFL61 without insert. Top trace: tRNA nucleosides from MT-8 transformed with plasmid pFL61 with the human gene inserted. Arrows indicate the retention time of i^6A for the two traces.

i^6A (Fig. 2, arrow, bottom trace), whereas the presence of the cDNA-bearing construct pFL–hMOD5 resulted in the appearance of a peak at this position (Fig. 2, top trace). Using UV and MS, the identity of the peak as i^6A was verified (Table 2). The amount estimated from the UV peak was 146 pmol, and from the MS analysis 133 pmol, showing that the UV peak is pure i^6A.

The functional complementation was not complete, since canavanine resistance was not suppressed (Table 1). This could be explained if only a fraction of the *SUP7* tRNA molecules was modified with i^6A in the transformed cells, resulting in only partial suppression of the nonsense mutation in the arginine permease gene. In accordance, the level of i^6A was only about one-fifth of that in the H57 strain (data not shown).

Table 2
Identification of i^6A from yeast tRNA by MS and UV spectrometry. Fragmentation resulted in four ions of significant size, whose relative intensities are listed as percent of the largest ion. This fragment has the *m/z* expected from i^6A less the ribose moiety. The λ_{max} was acquired directly in the HPLC effluent, where the pH was 6.0

	Mass spectra *m/z* (%)				UV spectra λ_{max} (nm)
	336.16	204.13	148.07	136.07	
Standard	17	100	17	27	271
MT-8 + hMOD5	12	100	29	30	371

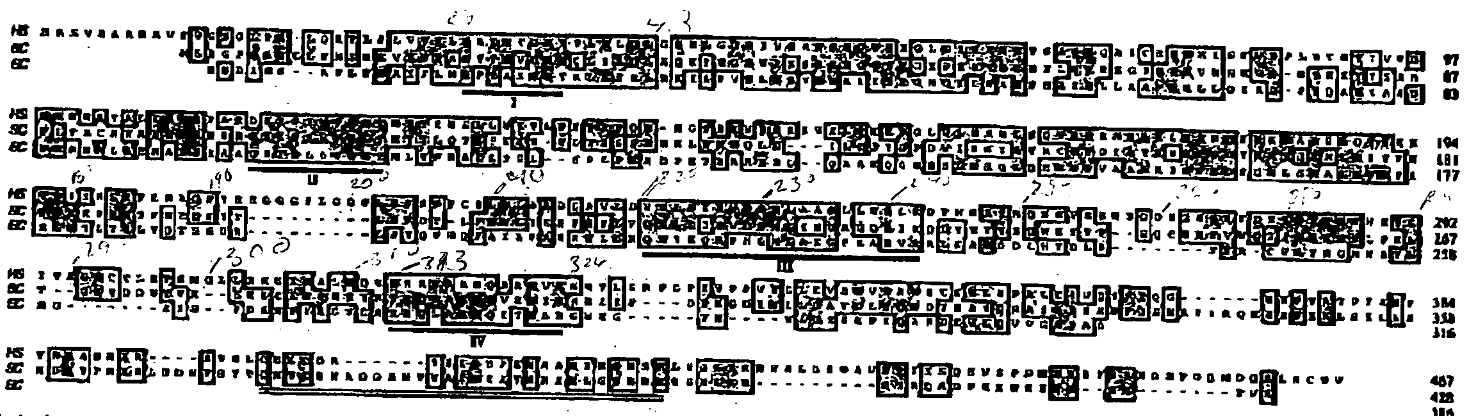


Fig. 3. Amino acid sequence alignment of IPTs from *Homo sapiens* (HS) (GenBank acc. no. AF074918), *S. cerevisiae* (SC) (GenBank acc. no. 2507067) and *E. coli* (EC) (GenBank acc. no. 1790613). Identical residues are boxed in dark gray and conserved substitutions are boxed in light gray. Motifs highly conserved among the three proteins are underlined and labeled I–IV. For explanations, see Section 3. The double-underlined region indicates a putative Zn-finger motif present in the two eukaryotic proteins.

A subnormal level of isopentenylation could be the result of low expression of the human gene, or that the protein has reduced activity in the yeast environment. The substrate specificity c uld be somewhat deviant from that of MOD5, since native substrate tRNAs might have idiosyncratic determinants for i⁶A modification. There is also a possibility of subcellular mislocalization of the human protein, for instance due to differences in localization signals (see below). Nevertheless, from the tRNA data it is clear that the cDNA encodes an IPT.

3.3. Sequence analysis of the human IPT

Fig. 3 shows the strong homology of the human IPT amino acid sequence with those of the IPT proteins of *S. cerevisiae* and *E. coli*. The conservation between the eukaryotic proteins is somewhat higher, 53% of overall similarity, than between either one of these and the bacterial protein, about 47% for each.

All well-conserved motifs present in tRNA isopentenyltransferases can be found in the deduced human protein sequence (motifs I to IV, Fig. 3). The best studied of these is an ATP/GTP motif A (P-loop, Saraste et al., 1990), located near the amino termini of the proteins (motif I, Fig. 3). ATP or GTP is not known to be required for the enzyme activity (Kline et al., 1969; Bartz et al., 1970; Rosenbaum and Gefter, 1972), but the *E. coli* MiaA has been found to bind several nucleoside di- and tri-phosphates, including ATP, strongly (Leung et al., 1997). Interestingly, this binding was strongly competitive with dimethylallyl pyrophosphate substrate binding, suggesting that the interaction might be a mechanism of regulation of the activity of this enzyme in vivo (Leung et al., 1997). The MiaA protein was found to be surprisingly abundant for a tRNA modifying enzyme, perhaps to counteract the strong inhibition by nucleotides (Leung et al., 1997). The high degree of amino acid conservation in the human protein sequence indicates that the nucleotide–dimethylallyl pyrophosphate interaction may have been preserved throughout evolution. The many duplicates found in the dbEST data bank also indicate that human IPT could be a fairly abundant protein.

Both human and yeast IPTs encode an additional ~100-amino-acid C-terminal extension compared with the bacterial protein (Fig. 3). The *S. cerevisiae* MOD5 protein has a classical bipartite nuclear localization signal (NLS) between amino acids 408 and 424, responsible for a nuclear pool of MOD5 protein (Tolerico et al., 1999). No homologous sequence at the corresponding position of the human IPT was found, but at least one good match to the bipartite NLS (KKGIEALKQVTKRYARK) was found at amino acids 308–325, partly overlapping a putative dimethylallyl pyrophosphate binding site (motif III, Fig. 3). Several clusters of basic residues reminiscent of known NLS were also found at positions 179–185 (PIIDKRKV) and 424–427/425–428 (KKRR/KRRR). It is thus possible that also the human IPT has a nuclear pool.

A search for other motifs disclosed a Zn-finger-like motif (amino acids 397–422, Fig. 3). A comparison with *S. cerevisiae* MOD5 showed the motif at the same relative position (amino acids 373–406, Fig. 3). Fig. 4 shows an alignment of these motifs with Zn-fingers of several proteins. For both IPT proteins, the motif conforms to the C2H2 class of Zn-finger first identified in the *Xenopus* transcription factor TFIIIA (Miller et al., 1985), but follows a distinct pattern C–x2–C–x(12,18)–H–x5–H. The closest match is found in the murine RNA-binding protein ZFR (Fig. 4). The Zn-finger-like motif was also found in three putative IPT genes turned up by BLAST searches of the genomic sequences of *S.*

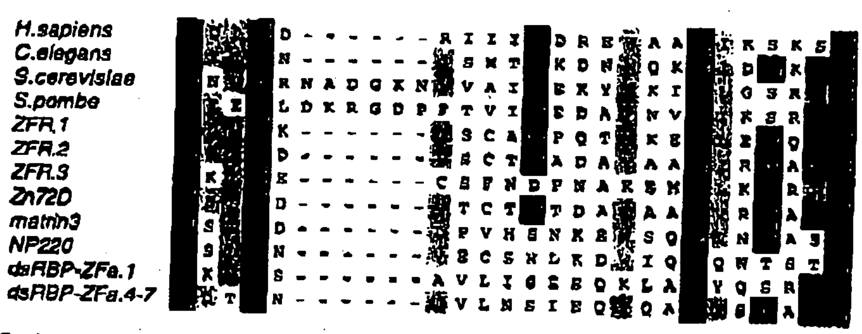


Fig. 4. Alignment of Zn finger motifs. The putative Zn fingers from the two eukaryotic IPTs of Fig. 2 and from two IPT homologues from *Caenorhabditis elegans* (CE) (GenBank acc. no. U13642) and *Schizosaccharomyces pombe* (SP) (from cosmid-C24J from the Sanger center (http://www.sanger.ac.uk/) are aligned to each other and to eight other amino acid sequences containing zinc fingers. The predicted Zn ligands are indicated. ZFR.1–3 are the three zinc fingers of *Mus musculus* ZFR (GenBank acc. no. AF071059) (Meagher et al., 1999). Zn72D is the third zinc finger of the *Drosophila melanogaster* homologue of ZFR (GenBank acc. no. U73125) (Meagher et al., 1999). Matrin 3 (GenBank acc. no. M63485) and NP220 (GenBank acc. no. D83033) are zinc fingers from two homologous nuclear matrix proteins from *Rattus norvegicus* and *H. sapiens* respectively. dsRBP-ZFa.1 and dsRBP-ZFa.4–7 are the first and the consensus of the four last Zn fingers respectively from the *Xenopus laevis* dsRBP-ZFa (GenBank acc. no. AF005083) (Finerty and Bass, 1997). Numbers given are relative to the second cysteine putative zinc ligand.

pombe and *C. elegans* (Fig. 4), and *Arabidopsis thaliana* (not shown). The motif thus appears to be a highly conserved feature of eukaryotic IPTs.

The Zn-finger-like motifs from the four eukaryotic homologues (Fig. 4) reveal a high degree of conservation of certain residues, most notably glycine and tryptophan (in *S. pombe* this tryptophan residue is substituted conservatively by phenylalanine). The spacing of the putative zinc-binding ligands is 12 amino acids in both the human and the *C. elegans* homologues, and 18 amino acids in the two yeast counterparts. Within the 12 residues stretch between the cysteines and histidines, Zn-fingers generally possess a conserved aromatic residue at the fourth position (+4) and a hydrophobic residue at the tenth position (+10). The Zn-fingers from all four MOD5 homologues in Fig. 4 show a reversal of the positions of these residues with hydrophobic amino acids at +4 (+10 in the yeast proteins) and aromatic at +10 (+16 in the yeast proteins).

The IPT Zn-finger motif is present in a single copy, an unusual but not unique feature. Similar domains have been found in proteins from both prokaryotes and eukaryotes and they may be important for protein–RNA interactions (Klug and Rhodes, 1987). However, it is also conceivable that the Zn-finger motifs of the eukaryotic IPTs are not involved in RNA interactions, like binding of the tRNA substrate, but instead might function as a nuclear retention signal (LaCasse and Lefebvre, 1995) or to stabilize enzyme conformation (Ke et al., 1988; Fourmy et al., 1993; Chong et al., 1995).

MOD5 of *S. cerevisiae* codes for two isoforms of the protein. One form has an 11 amino acids extension with a mitochondrial targeting signal. The second form is translated starting from a second ATG on the 3′ side of the signal-containing stretch (Boguta et al., 1994). The human cDNA contained a single ATG at position 15 (Fig. 1). Nevertheless, the N-terminal showed a complete absence of acidic, and an enrichment of hydrophobic, amino acids, characteristic of mitochondrial targeting signals. Although a classic amphiphilic α-helix (von Heijne, 1986) cannot be predicted for the first 18 residues, a segment with a very high hydrophobic moment was found between amino acids 10 and 21 (Fig. 3), indicating a possible mitochondrial targeting structure.

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